MOLECULAR PATHOGENESIS OF LIVER FIBROSIS

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ABSTRACT

Hepatic fibrosis is the final common pathway for most chronic liver diseases. The cell responsible for hepatic fibrosis appears to be the activated myofibroblast. The myofibroblast may be derived from quiescent hepatic stellate cells, epithelial to mesenhymal transition, or derived from bone marrow precursors. Studies in primary cultures of myofibroblasts and in mouse models of hepatic fibrosis have revealed several common pathophysiological mechanisms. Hepatic fibrosis is strongly associated with oxidative stress, increased transforming growth factor beta, hepatocyte death, and chronic inflammation. Finally, the reversal of fibrosis depends upon the elimination of the activated myofibroblast.

Most chronic liver diseases follow a common course. Chronic liver diseases progress from mild inflammation, to more severe inflammation, to fibrosis, and finally to cirrhosis. This is true in the most important liver disease in the developed world in the 20th century, which is hepatitis C infection and in what will be the most important liver disease in the 21st century, non-alcoholic fatty liver disease. Although each disease is characterized by progression from inflammation to fibrosis, the relationship between these two pathological conditions is not well understood.

The cell responsible for the fibrosis in all chronic liver diseases appears to be the activated myofibroblast. Currently, the origin of the activated myofibroblast is unresolved, and several cells potentially can fulfill this role. There is some evidence that bone marrow-derived fibrocytes or circulating mesenhymal cells can migrate through the injured liver and become myofibroblast to participate in the fibrotic process (1). Alternatively, there is a limited amount of evidence that hepatocytes, cholangiocytes, or even endothelial cells may undergo a transition to mesenhymal cells to become an activated myofibroblast.

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For hepatocytes and cholangiocytes, this process is called the epithelial-to-mesenhymal transition or EMT. Finally, the resident cells in the liver may be activated to become myofibroblasts. This can either be the tissue fibroblast located in the portal tract of the liver or the quiescent hepatic stellate cell (HSC) located in the Space of Disse. The predominance of evidence supports the role of the quiescent HSC activating to a myofibroblast and then producing the fibrous scar found in chronic liver diseases. Specific markers for stellate cells enable their identification in purified cell samples as well as in histological slides. These markers include glial fibrilar-associated protein (GFAP), nerve growth factor receptor p75, and synamin.

In the progression of chronic liver entry to fibrosis, all hepatic cells undergo specific changes. The hepatocytes are injured and they undergo apoptosis. The sinusoidal endothelial cells undergo a loss of fanestrae that is termed acapillarization of the sinusoids. The resident macrophage in the liver, the Kupffer cell, activates and produces a variety of chemokines and cytokines. Lymphocytes infiltrate the injured liver and contribute to the inflammation. Finally, the quiescent stellate cells are activated to produce extracellular matrix proteins.

The quiescent HSC is the major storage site of retinoids in the body. It also expresses neural markers as described above. When the HSC is activated, it loses its retinoid and starts expressing new receptors such as the platelet derived growth factor (PDGF) receptor and transforming growth factor-β (TGF-β) receptor. It also expresses new proteins such as α- smooth muscle actin. The activated HSC proliferates and synthesizes extracellular matrix proteins to produce the fibrous scar. To the best of my knowledge, the only pathway to eliminate the activated HSC is through apoptosis or senescence.

Studies over the past ten years have revealed multiple characteristics of HSCs. In addition to the production of ECM proteins stimulated by TGF-β and their proliferation stimulated by PDGF, the HSCs also contribute to other facets of liver pathophysiology. Driven by endothelian 1, the HSCs contract and may contribute to portal hypertension. Through the production of the metalloproteinase, MMP2, the HSCs contribute to the degradation of the normal extracellular matrix. Through the production of reactive oxidative species generated by NADTH oxidase, the HSCs contribute to oxidative stress. Through the production of chemokines and cytokines such as MCP-1, HSCs contribute to the chemotaxis of leukocytes as well as their own chemotaxis. Finally, the HSCs are the major source of the primary mytogen for hepatocytes, hepatocyte growth factor (HGF).

There are two types of studies that have contributed enormously to

the understanding of HSC biology. The first type of study uses primary cultures of HSCs. HSCs may be purified to homogeneity from rat, mouse and human liver specimens. This is because quiescent HSCs are the major storage site of retinoids, and their retinoid lipid droplets make HSCs by far the most buoyant cell in the liver. Therefore, when liver cells are placed on a density gradient, the HSCs band at the top of the gradient and are identified by their Vitamin A autofluorescence. After culture on plastic or type I collagen, the HSCs undergo an activation process very similar to their activation in vivo. They express new markers such as smooth muscle α-actin; they lose their retinoid; and they start synthesizing large quantities of extracellular matrix proteins.

The second type of experiment that has provided new insight into HSC biology is to induce hepatic fibrosis. These models of liver fibrosis were first developed in rats and then adapted to mice to take advantage of mouse genetics. These models include carbon tetrachloride, which is a model of post-necrotic fibrosis; thioacetamide, dimethelnitrosamine (DMN), bile duct ligation, which is the model for secondary biliary fibrosis; intragastric ethanol, heterologous serum, which is the model of autoimmune hepatitis; and methionine choline deficient diet, which is the model of non-alcoholic fatty liver disease. These different models of hepatic fibrosis have been used to study a variety of transgenic mice, including comparing gene knock out mice to their wild type litter mates. When the knock out mice have increased fibrosis compared to their wild type litter mates, the gene is protective, since eliminating the gene increased the fibrosis. When the knock out mice have decreased fibrosis compared to their wild type litter mates, the gene is considered to be fibrogenic, because eliminating the gene decreases fibrosis (Table 1). Using these studies, several critical generalizations can be drawn.

The first is that oxidative stress induces liver fibrosis. There are many potential sources of oxidative stress in the injured liver. These include the cytochrome P450IIE1 enzyme in the hepatocyte, which is induced by ethanol. The phagocytic NADPH oxidase in the Kupffer cell is another potential cell. Finally, our laboratory has demonstrated that the hepatic stellate cell itself expresses NADPH oxydase to produce reactive oxidative species leading to fibrosis (2). Examples of knock out mice that are resistant to hepatic fibrosis include the NADPH oxidase p47 subunit deletion and the angiotensin R1A deletion. Recent studies have demonstrated that the entire renin angiotensin pathway is present in the injured liver. The increased angiotensin II that binds to its AT1 receptor in the hepatic stellate cell leading to the activation of

TABLE 1
Modifiers of Hepatic Fibrosis Using Knock Out Mice

Increased "Protective"*	Decreased "Fibrogenic"**
IL-6, IL-10	TNFR1, TLR4, CD14
iNOS	Angiotensin R1a
Plasminogen	NADPH oxidase
Interferon γ	TGFβ1, Smad3, MMP13
Adiponectin	P21
Telomerase	FGF1/2, FGFR4
Bcl-xL	Leptin, OB-R
SOCS1 (+/−)	Dopamine βhydroxylase
Keratin	FasL, Cathepsin B
	C5

* Increased "Protective" - means the knock out mice have increased fibrosis and, therefore, the gene is protected from fibrosis

** Decreased "Fibrogenic" - means the knock out mice have decreased fibrosis and, therefore, the gene is fibrogenic

NADPH oxidase with subsequent HSC activation that leads to hepatic fibrosis. There is new enthusiasm for examining this pathway because of the potential therapeutic intervention with angiotensin converting enzyme inhibitors (ACE inhibitors) and angiotensin receptor blockers (ARBs). These drugs might be effective in preventing the progression of hepatic fibrosis.

The second major generalization is that TGF-β is required for liver fibrosis. TGF-β is the most potent fibrogenic cytokine. TGFβ induces fibrosis through multiple mechanisms, including direct activation of HSCs, stimulating synthesis of multiple ECMs, inhibiting ECM degradation by stimulating the production of tissue inhibitors of metalloproteases (TIMPs), and increasing its own synthesis through the AP-1 site on its own gene. Examples of knock out mice that are resistant to fibrosis have deletions in the genes for TGF-β1, SMAD3 and MMP13 (3).

The third generalization is that hepatocyte loss induces hepatic fibrosis. In other words, blocking normal liver regeneration will result in liver fibrosis. Thus, it appears that the primary response to injury will be liver regeneration, but if this is blocked, the default mode would be liver fibrosis. If hepatocytes undergo apoptosis without compensatory proliferation, fibrosis again would result. Therefore, it is informative that inhibiting hepatocyte apoptosis, such as with pancaspase inhibitors, has been shown to inhibit fibrosis in mouse models. Of clinical interest is that hepatocyte apoptosis correlates with fibrosis in patients with hepatitis C and NAFLD. Thus, noninvasive serum monitoring of hepatocyte apoptosis may provide insight into ongoing liver

fibrosis. Examples of knock out mice that are sensitive to fibrosis because they undergo more hepatocyte injury have deletions in the genes for telomerase, Bcl-xL and adiponectin. Examples of mice that are resistant to apoptosis because of deletions of pro-apoptotic genes have deletions of Fas and cathepsin B. These mice are resistant to hepatocyte apoptosis and, therefore, are resistant to hepatic fibrosis. The current hypothesis relating hepatocyte apoptosis to fibrosis is that when hepatocytes undergo apoptosis they produce apoptotic bodies. These apoptotic bodies are phagocytosed by Kupffer cells, hepatic stellate cells and hepatocytes. The phagocytosis results in the production of chemokines and cytokines, including MIP-2, KC and TGF-β1, which in turn activate hepatic stellate cells leading to fibrosis.

The fourth generalization is that chronic inflammation leads to fibrosis. In other words, injury to the liver with the production of down stream inflammatory mediators results in the activation of hepatic stellate cells and subsequent fibrosis. Examples of knock out mice that are resistant to fibrosis because they have less inflammation include those with gene deletions of TNF-α, TLR4, CD14 and LBP [4]. Despite the close association of inflammation with fibrosis, there is little known about the cross-talk between these two pathways in intracellular signal transduction. For example, TLR4 is activated by lypopolysaccharide (LPS) resulting in activation of NFB and of IRF3, which results in the transcription of inflammatory mediators. However, there is no cross-talk with the TGF-β pathway that results in the activation of SMADs 3 and 4 leading to induction of TGF-β responsive genes. Our laboratory has recently demonstrated that TLR4-deficient mice are remarkably resistant to hepatic f fibrosis using multiple models. The key pathway is mediated by LPS binding to TLR4 on hepatic stellate cells. This results in the down-regulation of Bambi, a pseudo receptor for TGF- β. When Bambi is down-regulated, the resultant TGF-β1 that is largely produced by Kupffer cells can then bind to the TGF-β receptor resulting in signal transduction and fibrogenesis. TLR4 has several mechanisms by which it will encourage fibrogenesis. TLR4 is known to be a major producer of chemokines and cytokines, which will recruit inflammatory cells and hepatic stellate cells to the injured liver. Furthermore, TLR4 will down-regulate the inhibitor Bambi and therefore allow for effective signal transduction of TGF-β. Recently, a whole genome array was conducted on patients with hepatitis C comparing patients who have mild fibrosis with patients who have extensive fibrosis. One of the first genes to be revealed by this analysis was TLR4. As predicted from our studies, patients with a TLR4 single nucleotide polymorphism (SNP) that leads to efficient signal transduc-

tion have increased hepatic fibrosis. Patients with SNPs in TLR4 with lower signal transduction of LPS are protected from hepatic fibrosis.

The final generalization is that apoptosis of hepatic stellate cells prevents and reverses fibrosis. The concept is that the activated HSC can be eliminated from the injured liver through apoptosis. This will remove the fibrogenic cell and allow for the normal degradation of the fibrous scar. The knock out mouse that has demonstrated this effect has the CEBP-β gene deletion, since CEBP- β is required to prevent apoptosis of activated hepatic stellate cells. Drugs that selectively induce apoptosis of activated HSCs have been demonstrated to reverse liver fibrosis, including gliotoxin, sulfasalazine and anti-TIMP1 antibody. This represents a paradigm shift in our understanding of liver fibrosis, since classic teachings have emphasized that cirrhosis is irreversible. Now we have examples of reversal of extensive hepatic fibrosis in models of liver fibrosis which are accompanied by loss of activated HSC. During resolution of fibrosis, there is apoptosis of activated HSCs. The inhibitors of collagenases TIMPs are produced by HSC and, therefore, are no longer present. This allows the collagenase to express their enzyme activity and degrade the ECMs in the fibrous scar. As predicted from these rodent models of hepatic fibrosis, increasing evidence has demonstrated that removing the known stimuli of fibrogenesis reverses fibrosis in patients. For example, treating autoimmune hepatitis with corticosteroids stops the inflammation and reverses the fibrosis. Patients with hemochromatosis who undergo phlebotomy to remove the excess iron can reverse their fibrosis. Patients with biliary obstruction who undergo decompression may reverse the progression of secondary biliary fibrosis. The most extensive studies have demonstrated the reversal of fibrosis with successful treatment of hepatitis C and hepatitis B. Finally, recent studies have demonstrated that treating the metabolic syndrome, such as with gastric bypass surgery, can reverse the steatosis, inflammation and fibrosis ([5]).

REFERENCES

1. Kisseleva T, Uchinami H, Feirt N, Quintana-Bustamente O, Segovia JC, Schwabe RF, Brenner DA. Bone marrow-derived fibrocytes participate in pathogenesis of liver fibrosis. J Hepatol 2006;45:4290–38.
2. DeMinicis S, Seki, E, Oesterreicher C, Schnabl B, Schwabe RF, Brenner DA. Reduced nitotinamide adenine dinucleotide phosphate oxidase mediates fibrotic and inflammatory effects of leptin on hepatic stellate cells. Hepatology. 2008;48:2016–26.
3. Hellerbrand C, Stefanovic B, Giordano F, Burchardt ER, Brenner DA. The role of TGFbeta1 in initiating hepatic stellate cell activation in vivo. J Hepatol. 1999;30:77–87.

4. Seki B, Brenner DA. Toll-like receptors and adaptor molecules in liver disease: update. Hepatology. 2008;48:322–35.
5. Schnabl B, Scholten D, Brenner DA. What is the potential role of antifibrotic agents for the treatment of liver disease? Nat Clin Pract Gastroenterol Hepatol. 2008;5:496–7.

DISCUSSION

Goldfinger, Boston: This very last slide reminds me of what Franklin Hanger, a hepatologist I studied under a long time ago at P&S, said. He said that alcoholic cirrhosis is a disease for which many are called but few are chosen.

Brenner, San Diego: Let me add something to that. I was taught that the increased intestinal permeability and the LPS in the portal blood was a late manifestation. So the change in permeability that we are proposing is actually a very early change in liver disease. So that would be different than our original ideas about alcoholic liver disease.

Berl, Denver: I was intrigued by the renin angiotensin systemic effect. Is that mediated by decreasing TGF beta? Have you looked at AT1 receptor in the animals? And the third question is, I am intrigued to see AQP2 on your gene chart, which is a vasopressin-dependent ion channel. I didn't even know it existed in the liver.

Brenner, San Diego: This study was a complete genome search by the company Celera. They had no preconceived hypothesis. They just had a lot of sequencing machines with nothing to do, and they screened a large number of patients. But the answer to your first question is, although it's not as dramatic as in the kidney, the angiotensin II activates its AT1 receptor in the stellate cell to induce TGFβ production. That is absolutely right, and I think that is a very important take-home message.

Keefe, Palo Alto: David, what about on the other side here? One of the fascinating lessons of anti-viral therapy for hepatitis C and hepatitis B has been reversibility of advanced fibrosis and even early cirrhosis that we did not think was the case. That is one of the fascinating stories, because all of us learned in the early days that cirrhosis was not reversible. Obviously, it is. So maybe I can let you extend your lecture a few slides.

Brenner, San Diego: That's right. So the concept is that if you remove the underlying stimulus in any way, and the most effective way that we know, hepatitis B is even more dramatic than hepatitis C, because you can drop the hepatitis B by 4 logs in days. Then you can remove the fibrogenic stimulus. It is very well-documented that any time you can remove the fibrogenic stimulus, you can get remarkable reversal of fibrosis, and that seems to be mediated by the activated stellate cells undergoing apoptosis. I am not quite sure why, but when we were taught this subject, it was not appreciated. However, it is very obvious in rodent models and has been reconfirmed again in virtually everything from secondary biliary cirrhosis, hepatitis C, and we had a recent paper where we showed that after gastric bypass, you can completely reverse the fibrosis in non-alcoholic fatty liver disease.

Keefe, Palo Alto: As you know, we can down-stage fibrosis by often two stages, like from early cirrhosis stage IV down to stage II. So it's really quite dramatic. But the exact mechanism, David, is what?

Brenner, San Diego: We really think that the phenomena you have observed is correct. You remove the stimulus, and the activated hepatic stellate cells undergo apoptosis. When the activated hepatic stellate cells are eliminated, the major source of TIMPs, which are tissue inhibitors of metalloproteinases, is removed, because TIMPs are produced by the activated hepatic stellate cells. Removing the TIMPS reveals the underlying collagenases, and the underlying collagenases can then degrade the fibrous

scar and revert to a more normal liver. So it seems like TIMPs are critical, and in collaboration with Bayer Pharmaceuticals, we did a study with an anti-TIMP antibody in a rodent model and got remarkable reversion of fibrosis. Therefore, even in the presence of the fibrogenic stimulus, carbon tetrachloride, in the absence of TIMPs, you can reverse the fibrosis.

Abboud, Iowa City: This is just a very brief question. Is angiotensin generated within the hepatocytes or the stellate cells?

Brenner, San Diego: The source of the angiotensin is believed to be the hepatic stellate cells. They seem to have a lot of the pathways required, when they are activated, for the whole rennin angiotensin system, which is a recent finding. That's not the systemic RES system that we know about. This is a local endogenous system. There is also good evidence that the entire pathway is present in kidneys and in hearts during fibrotic processes.

Alexander, Atlanta: Beautiful story and thank you very much for sharing it with us. I was struck by the similarity of the fibrotic stories in multiple organs, including lung, as has been so elegantly shown. This is beautiful. I was curious also about the renin-angiotensin system of which there are many tissues that have the complete system and have, in your models, you tried something as simple as ATR1R receptor blockers?

Brenner, San Diego: Yes, and the result is that you prevent fibrosis, as you predicted.